

Regular Article

Transcellular Transport of Creatinine in Renal Tubular Epithelial Cell Line LLC-PK₁

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Summary: Background/Aim: Creatinine is excreted into urine via tubular secretion in addition to glomerular filtration. In the present study, characteristics of the creatinine transport in renal epithelial cells were investigated.

Methods: The transcellular transport and accumulation of [¹⁴C]creatinine and [¹⁴C]tetraethylammonium (TEA) were assessed using LLC-PK₁ cell monolayers cultured on porous membrane filters.

Results: [¹⁴C]Creatinine was transported directionally from the basolateral to apical side of LLC-PK₁ cell monolayers. Basolateral uptake of [¹⁴C]creatinine was dependent on membrane potential, and was saturable with apparent K_m and V_{max} values of 13.2 ± 2.8 mM and 13.1 ± 3.1 nmol/mg protein/5 min, respectively. Concomitant administration of organic cations (1 mM) such as cimetidine, quinidine and trimethoprim inhibited both the transcellular transport and accumulation of [¹⁴C]creatinine. Furthermore, apical excretion of [¹⁴C]creatinine was not dependent on acidification of the apical medium.

Conclusions: Creatinine was subjected to directional transport across renal epithelial cells from the basolateral to apical side. The organic cation transporter should be involved in the basolateral uptake of creatinine.

Key words: creatinine; LLC-PK₁; organic cation transporter; tetraethylammonium; transcellular transport; tubular secretion

Introduction

Creatinine (M.W.: 113.12, pKa: 4.8, 9.2) is a catabolic product of creatine, with both positive and negative charges, i.e. a zwitterion, at physiological pH. Because creatinine is excreted mostly into urine, its systemic clearance has been used for the evaluation of kidney function. Although the renal disposition of creatinine is mainly mediated by glomerular filtration, the secretion and reabsorption of creatinine at renal tubules have been also recognized.^{1,2)} The secretory fraction of creatinine is significant especially in patients with glomerular disorders,³⁾ and causes an overestimation of the glomerular filtration rate (GFR). Although organic ion transport systems have been implicated in the tubular secretion of creatinine, there is no evidence to reveal the transcellular transport of creatinine across the renal tubular epithelium.

In the proximal tubules of the kidney, organic ion transporters mediate elimination of cationic drugs into

the urine.⁴⁻⁷⁾ According to studies using isolated membrane vesicles^{8,9)} and cultured renal epithelial cells,^{10,11)} the basolateral uptake of tetraethylammonium (TEA) is driven by the transmembrane potential difference. Subsequently, TEA is excreted across apical membranes by the H⁺/organic cation antiporter. LLC-PK₁ cells, an established epithelial cell line derived from pig kidney, retain characteristics of the proximal tubular epithelium, and therefore they have been used for studying the tubular transport of various solutes including organic cations.¹⁰⁻¹³⁾ We demonstrated that the transcellular transport of TEA across LLC-PK₁ cell monolayers was directional from the basolateral to apical side, and was stimulated markedly by acidification of the apical medium.¹¹⁾ In the present study, we characterized creatinine transport in renal epithelial cells LLC-PK₁ cultured on porous membrane filters.

Methods

Cell Culture: LLC-PK₁ cells, obtained from the

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American Type Culture Collection (ATCC CRL-1392; Rockville, MD, USA), were grown on plastic dishes in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Thermo Trace, Victoria, Australia) without antibiotics in an atmosphere of 5% CO₂/95% air at 37°C.¹⁸⁾ For transport experiments, the cells were seeded on microporous membrane filters (3- μ m pore, 4.71-cm² growth area) inside Transwell® cell culture chambers (Costar, Cambridge, MA, USA) at a density of 5×10^5 cells/cm². The cell monolayers were used for transport experiments at 6 days after seeding. In this study, LLC-PK₁ cells between passages 213 and 223 were used.

Uptake Experiments with LLC-PK₁ Cells: The transport of [¹⁴C]creatinine and [¹⁴C]TEA by LLC-PK₁ cells was measured using cell monolayers grown in Transwell® cell chambers (Costar).¹¹⁻¹³⁾ [³H]D-Mannitol was used to calculate paracellular fluxes and the extracellular trapping of [¹⁴C]creatinine and [¹⁴C]TEA. The incubation medium for the uptake experiments contained: 145 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM D-glucose and 5 mM HEPES (pH 7.4). The composition of the high K⁺ incubation medium was 3 mM NaCl, 145 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM D-glucose and 5 mM HEPES (pH 7.4). The pH of the medium was adjusted with NaOH or HCl. After removal of the culture medium from both sides of the monolayers, the cells were washed once with 2 mL of incubation medium in each side for the 4.71-cm² chamber and then incubated for 10 min at 37°C with 2 mL of the same medium in each side. The medium was replaced with 2 mL of incubation medium containing [¹⁴C]creatinine or [¹⁴C]TEA in either the apical or basolateral side and the cells were incubated at 37°C. The incubation medium without substrates was added to the opposite side. The medium was immediately aspirated off and the culture inserts were rapidly rinsed twice with 2 mL of ice-cold incubation medium in each side. The cells were solubilized in 0.5 mL of 0.5N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford,¹⁴⁾ using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine γ -globulin as a standard. For the *cis*-inhibition experiment, the uptake of [¹⁴C]creatinine was achieved by adding various concentrations of unlabeled inhibitors to the incubation medium.

Materials: [2-¹⁴C]Creatinine hydrochloride (55 mCi/mmol) and [ethyl-1-¹⁴C] tetraethylammonium bromide (55 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). D-[1-³H(N)]Mannitol (17 Ci/mmol) were obtained from PerkinElmer Life Science Products (Boston, MA,

USA). Unlabeled creatinine, cimetidine, tetraethylammonium bromide, (\pm)-chlorpheniramine maleate, quinidine, guanidine hydrochloride, salicylic acid and p-aminohippuric acid were obtained from Nacalai Tesque (Kyoto, Japan). 1-Methyl-4-phenylpyridinium iodide, N¹-methylnicotinamide iodide and probenecid were purchased from Sigma-Aldrich. Trimethoprim was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other compounds used were of the highest purity available.

Statistical Analyses: Data were analyzed statistically with one-way analysis of variance followed by Dunnett's test. P values of less than 0.05 were considered to be significant.

Results

We measured the transepithelial flux and intracellular accumulation of creatinine across LLC-PK₁ cell monolayers grown on porous membrane filters, in comparison with the transport of tetraethylammonium, TEA, a typical organic cation (**Fig. 1**). The basolateral-to-apical transport of [¹⁴C]creatinine and [¹⁴C]TEA was much greater than the apical-to-basolateral transport, and its rate was nearly constant for up to 60 min (**Figs. 1A and 1C**). The cellular accumulation of [¹⁴C]creatinine and [¹⁴C]TEA from the basolateral side after the transport experiments for 60 min was 2.0- and 3.6-fold greater than that from the apical side, respectively (**Figs. 1B and 1D**). These results suggested that creatinine was subjected to directional transport across LLC-PK₁ cell monolayers, corresponding to the renal tubular secretion.

Figure 2 shows the time-course and concentration-dependence of the accumulation of [¹⁴C]creatinine from the basolateral side of LLC-PK₁ cell monolayers. The accumulation was linear for up to 5 min (**Fig. 2A**). Furthermore, [¹⁴C]creatinine accumulation for 5 min was saturated at high concentrations (**Fig. 2B**). After subtracting the nonspecific component of [¹⁴C]creatinine accumulation in the presence of 5 mM 1-methyl-4-phenylpyridinium, the mean \pm S.E. of the apparent Michaelis constant (K_m) and maximal uptake rate (V_{max}) were obtained from three separate experiments as 13.2 ± 2.8 mM and 13.1 ± 3.1 nmol/mg protein/5 min, respectively. Eadie-Hofstee plots (inset of **Fig. 2B**) for these experiments seemed to be linear, suggesting the involvement of a single transport system.

Next, we examined the effect of membrane potential on the accumulation of [¹⁴C]creatinine from the basolateral side. As shown in **Fig. 3**, the accumulation of [¹⁴C]creatinine and [¹⁴C]TEA from the basolateral side of LLC-PK₁ cell monolayers decreased in the presence of high K⁺ (145 mM) buffer, as did that of [¹⁴C]TEA. Furthermore, the accumulation of [¹⁴C]creatinine and [¹⁴C]TEA decreased in the presence of 10 mM

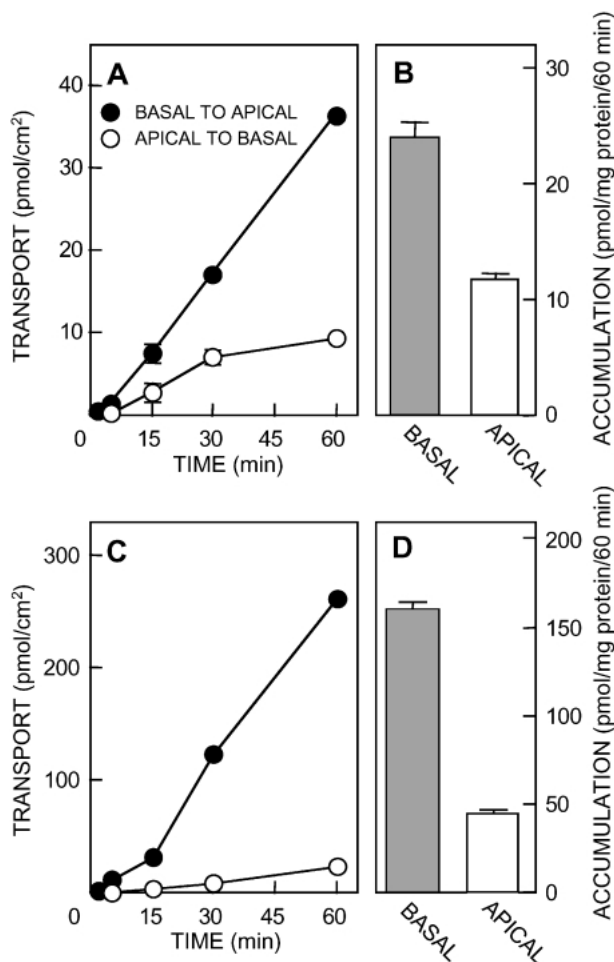


Fig. 1. Transcellular transport and accumulation of [^{14}C]creatinine (A and B) and [^{14}C]TEA (C and D) in LLC-PK $_1$ cell monolayers. LLC-PK $_1$ cells were incubated at 37°C with 5 μM [^{14}C]creatinine or 5 μM [^{14}C]TEA added to the basolateral (closed circle, pH 7.4) or apical (open circle, pH 7.4) side. The radioactivity on the opposite side was periodically measured (A and C). After a 60-min incubation, the radioactivity of solubilized cells was measured (B and D). Each point or column represents the mean \pm S.E. for three monolayers from a typical experiment. When error bars are not shown, they are included within the symbols (A and C).

Ba^{2+} , a nonselective K^+ channel blocker.¹⁸⁾

To investigate further the involvement of organic cation transporters in [^{14}C]creatinine transport in LLC-PK $_1$ cell monolayers, we evaluated the effects of concomitant organic ions (1 mM) added to the basolateral side on the transcellular transport and accumulation of [^{14}C]creatinine. In this experiment, the transport of [^{14}C]creatinine was measured for 15 min to obtain a sufficient amount of radioactivity in the apical chamber. As shown in **Fig. 4**, trimethoprim, cimetidine, quinidine, and 1-methyl-4-phenylpyridinium inhibited both the basolateral-to-apical transport and accumulation of [^{14}C]creatinine. Other organic cations such as TEA, chlorpheniramine, and N^1 -methylnicotinamide

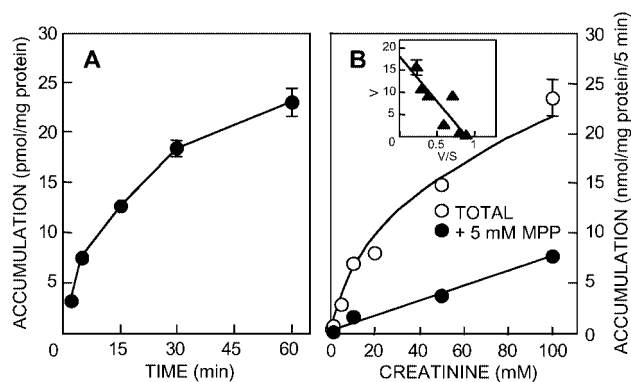


Fig. 2. Time course (A) and concentration-dependence (B) of [^{14}C]creatinine accumulation in LLC-PK $_1$ cell monolayers. A; LLC-PK $_1$ cells were incubated for the specified periods at 37°C with 5 μM [^{14}C]creatinine added to the basolateral side. The pHs of both apical and basolateral media were 7.4. Each point represents the mean \pm S.E. of three independent experiments. B; LLC-PK $_1$ cells were incubated at 37°C for 5 min with the various concentrations of [^{14}C]creatinine indicated in the absence (open circle) or presence (closed circle) of 5 mM 1-methyl-4-phenylpyridinium (MPP) added to the basolateral side. The pHs of both apical and basolateral media were 7.4. Each point represents the mean \pm S.E. for three monolayers from a typical experiment. *Inset*: Eadie-Hofstee plots of [^{14}C]creatinine uptake after a correction for nonsaturable components. V, uptake rate (nmol/mg protein/5 min); S, creatinine concentration (mM). When error bars are not shown, they are included within the symbols.

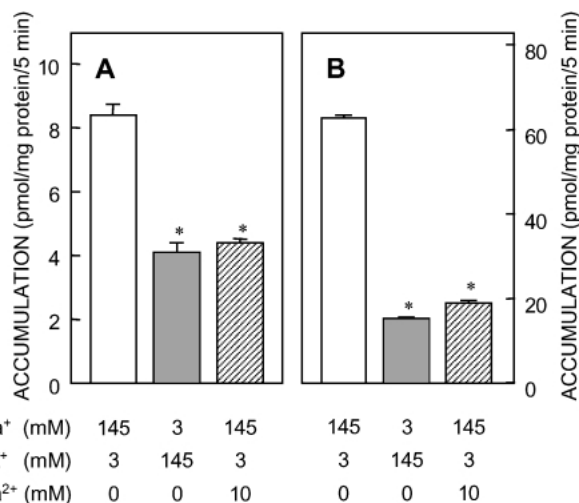


Fig. 3. Effect of membrane potential on uptake of [^{14}C]creatinine (A) and [^{14}C]TEA (B) from the basolateral side in LLC-PK $_1$ cell monolayers. LLC-PK $_1$ cell monolayers were incubated at 37°C for 5 min with incubation medium at the indicated ion concentrations on both sides (pH 7.4) with 5 μM [^{14}C]creatinine (A) or 5 μM [^{14}C]TEA (B) added to the basolateral side. Each column represents the mean \pm S.E. for three monolayers from a typical experiment. *, $P < 0.05$, significant difference from control using analysis of variance followed by Dunnett's test.

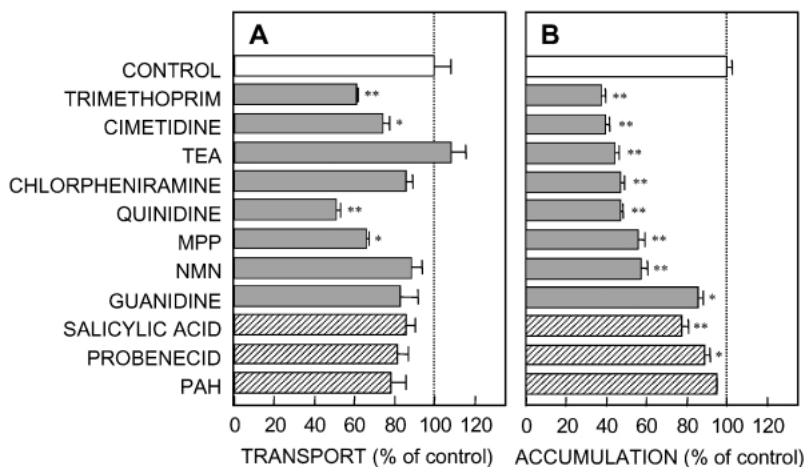


Fig. 4. Effect of organic cations and anions on the transcellular transport (A) and accumulation (B) of [^{14}C]creatinine in LLC-PK₁ cell monolayers. LLC-PK₁ cell monolayers were incubated at 37°C for 15 min with 5 μM [^{14}C]creatinine added to the basolateral side in the absence (control) or presence of cationic or anionic compounds (1 mM) on the basolateral side. The pHs of both basolateral and apical media were 7.4. MPP, 1-methyl-4-phenylpyridinium; NMN, N¹-methylnicotinamide; PAH, *p*-aminohippuric acid. Each column represents the mean \pm S.E. for three monolayers from a typical experiment. *, $P < 0.05$; **, $P < 0.01$, significant difference from control using analysis of variance followed by Dunnett's test.

also inhibited significantly the accumulation of [^{14}C]creatinine from the basolateral side (**Fig. 4B**). Furthermore, guanidine, salicylic acid, and probenecid slightly inhibited the accumulation of [^{14}C]creatinine from the basolateral side (**Fig. 4B**). In contrast, *p*-aminohippuric acid had little inhibitory effect on both the transcellular transport (**Fig. 4A**) and accumulation (**Fig. 4B**).

Finally, we examined the effect of apical pH on the transcellular transport and accumulation of [^{14}C]creatinine. Both the transcellular transport (**Fig. 5A**) and accumulation (**Fig. 5B**) of [^{14}C]creatinine were mostly independent of the apical pH. In contrast, the transcellular transport of [^{14}C]TEA increased markedly with the acidification of the apical medium (**Fig. 5C**), as demonstrated by us.¹¹ Consistent with the increased extrusion of [^{14}C]TEA from the cells across apical membranes, the accumulation of [^{14}C]TEA decreased (**Fig. 5D**).

Discussion

Although organic ion transporters have been implicated in the tubular secretion of creatinine,^{15,16} the characteristics of tubular secretion of creatinine have not been clarified. In the present study, we found that the transport of creatinine across LLC-PK₁ cell monolayers was directional from the basolateral to apical side (**Fig. 1**), and was markedly reduced in the presence of organic cations (**Fig. 4**), suggesting the involvement of organic cation transporters in the transcellular transport of creatinine. This is the first report to demonstrate the transcellular transport of creatinine *via* organic cation transport systems.

Several organic cation transporters (OCTs) have been

identified.⁵ hOCT2 is the dominant organic cation transporter in the human kidney,¹⁷ and is driven by differences in membrane potential, mediating the basolateral uptake of organic cations into the renal epithelial cells. Recently, we demonstrated that creatinine was specifically transported by hOCT2, but not hOCT1 in the cDNA transfected HEK293 cells.¹⁸ In the present study, the basolateral uptake of creatinine was dependent on the inside-negative membrane potential, being consistent with our previous findings. In addition, the apparent K_m value of the basolateral uptake of creatinine in LLC-PK₁ cells (13.2 ± 2.8 mM, **Fig. 2B**) was comparable to that in the hOCT2-expressing HEK293 cells (4.0 ± 0.3 mM).¹⁸ Furthermore, the ability of various organic ions to inhibit the cellular accumulation of creatinine (**Fig. 4**) suggested that the creatinine uptake in LLC-PK₁ cells is mediated by an OCT-like transporter expressed at the basolateral membrane.

Acidification of the apical medium did not stimulate the elimination of creatinine from the cells, in contrast to the marked stimulation of apical excretion of TEA (**Fig. 5**). These results suggested that the contribution of the H⁺/organic cation antiporter to the apical extrusion of creatinine is limited. Provided that the volume of LLC-PK₁ cells is 3.4 $\mu\text{L}/\text{mg}$ protein,¹⁹ the intracellular concentration of [^{14}C]creatinine after a 60-min incubation reached 7.4 μM (**Fig. 1B**), which was about 1.5-fold the extracellular concentration of [^{14}C]creatinine (5 μM). Taking into consideration that the accumulation of TEA was highly concentrative, i.e. 10-fold higher than that of extracellular [^{14}C]TEA (**Fig. 1D**), the transport of creatinine both at apical and basolateral

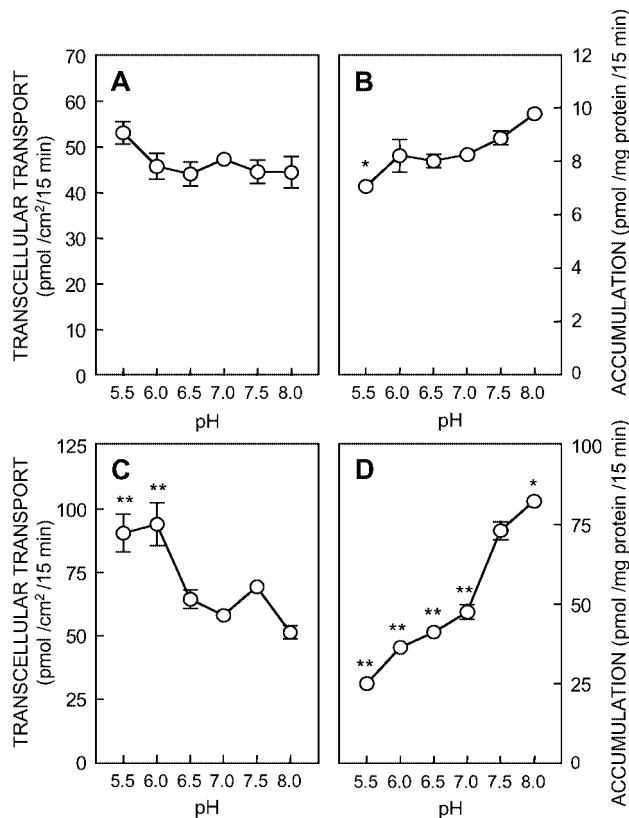


Fig. 5. Effect of apical pH on transcellular transport and accumulation of [14 C]creatinine (A and B) and [14 C]TEA (C and D) in LLC-PK₁ cell monolayers. LLC-PK₁ cell monolayers were incubated at 37°C for 15 min with 5 μ M [14 C]creatinine or [14 C]TEA added to the basolateral side and apical media of various pH (5.5–8.0). The pH of the basolateral medium was 7.4. The radioactivity in the apical medium (A and C) and solubilized cells (B and D) was measured after incubation. Each point represents the mean \pm S.E. for three monolayers from a typical experiment. *, $P < 0.05$; **, $P < 0.01$, significant difference from the value at pH 7.4 using analysis of variance followed by Dunnett's test. When error bars are not shown, they are included within the symbols.

membranes should be comparable with that of TEA.

In the present study, concomitant administration of cimetidine and trimethoprim significantly inhibited the creatinine transport across LLC-PK₁ cell monolayers (Fig. 4). It is reported that oral cimetidine administration improves the preciseness of GFR estimation due to the inhibition of tubular creatinine secretion in patients with renal diseases and renal transplant recipients.^{16,20} In another study by Berglund *et al.*,¹⁵ the serum creatinine level was elevated by a twice-daily administration of 160 mg of trimethoprim plus 800 mg of sulfamethoxazole through inhibition of the tubular secretion of creatinine. It is reported that a single oral dose of 200 mg of cimetidine to patients with normal renal function gave C_{\max} values of between 2.3 μ M and 6.8 μ M,²¹ which is lower than the IC₅₀ value of cimetidine for the uptake of creatinine by hOCT2 (27 \pm 6 μ M).¹⁸ On the

other hand, the IC₅₀ value of trimethoprim for the uptake of creatinine by hOCT2 (21 \pm 2 μ M) was marginal in view of the hOCT2-mediated transport of creatinine at therapeutic concentrations of trimethoprim.¹⁸ Considering that a higher dose of cimetidine (800 mg daily) is used for the treatment of gastric and duodenal ulcers and reflux esophagitis and the systemic clearance of cimetidine and trimethoprim is delayed in patients with decreased renal functions. Concomitant administration of these drugs would interfere in part with the tubular secretion of creatinine via hOCT2 and increase the serum creatinine level.

In conclusion, we have demonstrated for the first time that transport of creatinine across renal epithelial cells was directional from the basolateral to apical side. These observations are relevant for understanding the molecular mechanisms underlying the tubular secretion of creatinine.

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